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SECOND ANNUAL REPORT

Jordi Casals-Ariet, M.D.

January 1979

Supported by

U.S.Army Research and Development Command, Fort Detrick,
Frederick, Maryland 21701

Contract DADA-17-77-C-7035

Yale University School of Medicine
New Haven, Connecticut 06510

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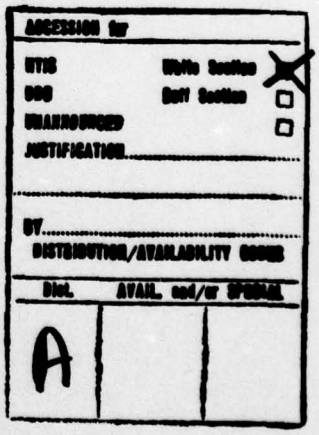

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20. Abstract (continued)

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Infective virus at high titers has been recovered from spot-slides prepared by the basic procedure used, acetone fixation; the possibility of preparing satisfactory avirulent slides by treatment of the infected cells with betapropiolactone is being investigated.

Inoculation of cell monolayers with either Junin or dengue type 2 viruses followed by daily processing of a sample of the monolayers and observation by indirect IF has permitted specific detection of small amounts of virus 1 to 3 days after inoculation, and from 2 to 4 days before cytopathic effect (CPE) was visible. The inference is that a similar procedure might result in acceleration of a specific diagnosis in a natural infection.

A model of infection using Banzi virus inoculated to mice has been developed, in which mice have a viremia of 4 to 5 days duration. Using the model as an analogy of arbovirus infection in nature, studies have been initiated to determine the prospect of IF for early and rapid diagnosis through detection of virus directly in the blood.

The successful use of IF with spot-slides for seroepidemiological surveys has continued with Lassa and Congo-Crimean hemorrhagic fever (CCHF) viruses. The same procedure has been applied to the detection of cases of laboratory-acquired infections with Pichinde virus and, for the first time, with Tacaribe virus.

SUMMARY

Slides with 12 small circular areas of cells infected with a virus, spot-slides, for use as antigen in the indirect immunofluorescence (IF) test have been prepared with the following viruses: chikungunya, dengue type 2, Naples phlebotomus fever, Western equine encephalitis (WEE) and yellow fever. Spot-slides containing a mixture of cells infected with different alphaviruses (group A), WEE, eastern equine encephalitis (EEE), Venezuelan equine encephalitis (VEE) and chikungunya were also prepared; hyperimmune antisera for group A viruses not present in the mixture gave positive reactions. It is anticipated that these and similar polyvalent slides will be useful in field situations.

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FOREWORD

In conducting the research described in this report, the investigator adhered to the "Guide for Laboratory Animal Facilities and Care", as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences - National Research Council.

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BODY OF REPORT

I. Statement of problem

The problem under consideration in this research project relates to methods of early and rapid diagnosis of arbovirus and arenavirus diseases of man. There is no doubt that early diagnosis of disease is desirable, if for no other reason because diagnosis influences prognosis. With arboviruses and arenaviruses a specific diagnosis is of great importance as it may determine or influence management and treatment of the patient, including degree of isolation and administration of specific immune plasma which is often in short supply; if chemotherapeutic preparations become available, their use may be influenced by the diagnosis. Diagnosis will also determine the advisability of applying public health measures, such as vector control, quarantine and, when available, vaccination. All these measures are expensive and disruptive; therefore, in order not to waste effort and resources, an early diagnosis is very important.

Having established the need for an early diagnosis, the second part of our problem is to choose the best procedure to achieve it. The most rapid diagnosis is accomplished by visualizing and specifically identifying the virus or its antigens in clinical specimens, with no passage and propagation in experimental hosts. In the event that direct observation of clinical specimens fails to give a diagnosis, isolation and identification of the virus in experimental host systems is attempted. If the virus is not detected in clinical specimens, either directly or indirectly, the diagnosis is attempted by detection of serum antibodies.

The main question addressed in this research project is to investigate the possibilities for early diagnosis of one of the new techniques, IF, and compare its results with those of other, longer established tests, complement fixation (CF), hemagglutination inhibition (HI) and neutralization; and to find out whether antigen-containing slides for IF can be prepared and preserved in reasonably large quantities with a number of selected arboviruses and arenaviruses, or mixtures thereof, to be used for early and rapid diagnosis and for seroepidemiological surveys.

II. Background

Specific diagnosis of a current arbovirus or arenavirus infection of man is generally made by isolation and identification of the virus, a process that under favorable circumstances can take several days; or by detection of antibody development between acute and convalescent serum samples.

The fastest diagnosis is accomplished by detecting the virus or viral antigens directly in clinical specimens by one of several techniques: electron microscopy, immunoelectron microscopy, IF, radioimmunoassay (RIA), enzyme linked immuno assays (Elisa or immunoperoxidose). Observation by IF of pharyngeal secretions, throat swabs, urinary sediments, corneal impressions and conjuctival scrapings has been variously applied to early diagnosis of respiratory viral infections, measles, rabies and Lassa fever (McCormick and Johnson, 1978); immunoelectron microscopy of fecal material has been used for diagnosis of rotavirus-caused gastroenteritis of man; electron microscopy of

sedimented blood has been applied to the detection of Ebola virus; and RIA has become a routine procedure for determining the presence of hepatitis B antigen in the blood. Among arboviruses, IF has been reported as successfully detecting Colorado tick fever virus in the blood of patients (Emmons and Lennette, 1966).

When the virus has not been observed directly in clinical specimens, diagnosis based on virus isolation can be speeded up by inoculating the suspect material into a susceptible host system, and attempting to identify the antigen in the host a short time after inoculation; identification of the antigen is generally done by IF. This procedure has been applied mainly to the diagnosis of dengue, either by inoculation of mosquitoes (Kubersky and Rosen, 1977) or cell cultures (Digoutte et al., 1979).

In the event that diagnosis based on virus identification or isolation is unsuccessful, serological tests are used. Detection of seroconversion, since it depends on obtaining a second serum sample, is a delayed diagnostic procedure; however, if a given virus is unlikely to have infected the patient in the remote past, a positive single serum with a high titer for that virus gives a valuable diagnostic lead. A presumptive diagnosis with an early single serum sample may be arrived at by fractionation of the immunoglobulins by centrifugation in a preformed gradient; virus antibodies associated with the IgM fraction indicate recent exposure to the virus; in addition, these antibodies are more type specific than the IgG.

Two elements must be considered in selecting an assay for early and rapid serological diagnosis; the time required to perform the test and the time in the course of the infection when the antibodies are detectable by the particular test chosen. With arboviruses, particularly alphaviruses (group A) and flaviviruses (group B) with which much information is available, the consensus is that antibodies are first detected by neutralization test, followed by HI and later by CF (Casals and Palacios, 1941; Lennette et al., 1953; Theiler and Casals, 1958; Buescher et al., 1959; Stallones et al., 1964; Galinovic-Weisglass and Vesenjak-Hirjan, 1976).

With respect to the arenaviruses, the neutralization test has not been generally used as an aid to a current diagnosis; the antibodies are late in developing, the execution of the test to the final reading takes a long time and the test itself presents certain difficulties, mainly with lymphocytic choriomeningitis (LCM) (Hotchin and Kinch, 1975) and Lassa fever (Monath et al., 1973). The CF test while easy to perform gives a delayed answer; for example, with LCM (Deibel et al., 1975) only 1 of a series of 7 patients was positive within the first 10 days after onset; and with Lassa fever, only 3 of 29 sera taken from patients between 3 and 14 days after onset were positive (Monath and Casals, 1975). In an extensive study of Bolivian hemorrhagic fever (BHF) patients (Johnson, 1970) it was shown that both CF and neutralizing antibodies were rarely found before the 20th day from onset; a similar situation appears to occur with Argentinian hemorrhagic fever (AHF). No HI test is applicable to these viruses for lack of an antigen.

The IF test has been increasingly applied, particularly since 1975, to the diagnosis of arbovirus and arenavirus diseases, especially the latter; among other viruses, the technique has been used with Colorado tick fever (Emmons et al., 1969), BHF (Peters et al., 1973), LCM (Deibel et al., 1975), AHF (Greber et al., 1975), Lassa fever (Wulff and Lange, 1975) and CCHF (Zgurskaya et al

1975). In addition to the intrinsic value of the test, two technological developments have promoted its use: commercial availability of extremely efficient incident light microscopes at reasonable prices; and development of multi-chambered microscope slides and teflon-coated water-repellent spot slides, which allow up to 12 serum samples to be processed simultaneously on a slide.

While this report is concerned only with the development and application of the IF test, at least two other serological techniques should be considered in the future for use in rapid diagnosis of arenavirus and arbovirus infections: radio-immuno-assay (RIA) particularly the solid phase one (SPRIA) and the enzyme linked immunosorbent assay (ELISA). The RIA has been used with arboviruses both in connection with detection of antigens (Dalrymple et al., 1972; Trent et al., 1976) and antibodies (Jahrling et al., 1978); in the latter case, protein A-bearing Staphilococcus aureus was used as the solid phase immunoadsorbent. Application of the technique to serological studies with LCM has been reported (Blechschmidt et al., 1977); it has been extensively used since 1971 for detection of antibodies for hepatitis antigens (Lander et al., 1971); and its use in the diagnosis of rubella, mumps, measles, herpesvirus and varicella-zoster has been reported (Forghani et al., 1976). The possibility of applying RIA to early diagnosis of arbovirus and arenavirus infections is being investigated in this laboratory (Converse and Casals, 1979). ELISA (Engvall and Perlmann, 1972) has been successfully employed for antibody titrations with several parasitic diseases (Voller et al., 1976) as well as with several viruses, including measles and cytomegalovirus (Voller and Bidwell, 1976); application of this technique to arboviruses is being explored in this laboratory (C. Frazier and O. Wood, 1978, personal communication).

III. Approach

The working hypothesis made was that a rapid diagnosis of arbovirus and arenavirus infections can be made through the use of the IF technique and prepared slides bearing the necessary antigens. IF was selected owing to the simplicity of the test once the slides are available and to the short time needed to execute the test, 2 to 3 hours.

To test the hypothesis of a rapid diagnosis by IF, it is necessary to have sera taken early in the disease; it was recognized that such sera are not easy to come by. For this reason our approach entails the use of combinations of laboratory animals and particular viruses such that, peripheral inoculation of virus to the animal results in an infection characterized by viremia followed either by disease and death, or by no apparent disease. Given these models of infection, sera collected daily will be tested by IF and other serological tests -- CF, HI, neutralization -- to determine which method gives the earliest diagnosis.

Another approach is to attempt to diagnose the infection by identification of the virus during the viremic phase by means of IF.

The efficacy of the IF test with spot-slides as developed during the first year of this research project will be investigated on a continuing basis by applying it to available human sera, mainly late convalescent or survey sera.

IV. Materials and Methods

Viruses. The viruses and strains employed were: chikungunya, Ross; EEE, Alabama 09-28-0930; VEE, TC 80; WEE, McMillan; Banzi, SA H336; dengue type 2, NGB; JE, Nakayama; Langat, TP 21; yellow fever, Asibi; Naples phlebotomus fever, Sabin; Junin, XJ high mouse passage; Pichinde, Cali An 3739; and Tacaribe, Tr 11573.

The viruses are maintained as a 10% suspension of infected newborn mouse brain tissue in 50% fetal calf serum in phosphate buffered saline (PBS), pH 7.2.

Cell cultures. The following cell cultures routinely maintained in this laboratory have been used: VERO, BHK-21, LLC-MK2, and CER (Smith et al., 1977). The cells are maintained in 150 cm^2 plastic bottles; transfers are made every 8-10 days for VERO and LLC-MK 2, or every 4-6 days for the others.

Animals. The following have been used: mouse (Mus musculus), Swiss outbred, Charles River CD (R) 1 strain; hamster (Mesocricetus auratus), LAK: OVG (SVR) strain; guinea pig (Cavia porcellus), Yale breed; and rabbit (Oryctolagus cuniculus), New Zealand strain. All animals were purchased from commercial dealers; their weights on delivery, shortly before they were used in an experiment, were: hamsters, 90 to 110 gr; guinea pigs, 400 to 500 gr; and rabbits 2000 to 2500 gr. Mice were used either as newborn, between 1 and 5 days old, or as adults. In the latter case, they were weaned in our own animal quarters and held until used; in this fashion their age was exactly known.

Bleeding of animals. Mice, hamsters and guinea pigs were bled by cardiac puncture under deep ether anesthesia. In experiments requiring serial bleeding mice and hamsters were bled out in groups of 3 each day, the bloods being pooled; rabbits were bled by nicking the marginal ear vein and collecting the drops of blood in a test tube. Guinea pigs were bled several times with intervals of 2 or 3 days; blood was individually kept.

Preparation of spot-slides. The protocol for preparation of the slides was described in detail in the first annual report, submitted on 5 April 1978. Because this operation was basic to the work reported here, the method is given in detail with slight modifications.

Microscope slides 75 x 25 mm, coated with Teflon except on 12 circular areas (spots) 5 mm in diameter are purchased from Cel-Line Associates, Minotola, New Jersey.

Cells are seeded in Corning 150 cm^2 plastic flasks, 8×10^4 to 1×10^5 cells/cm^2 or, approximately, 10^7 cells per flask. At the appropriate time depending on the cell -- 2 to 3 days for CER, 4 to 8 for the others -- the monolayers are inoculated with a virus in the form of a suspension of infected newborn mouse brain tissue at dilutions from 10^{-3} to 10^{-6}, depending on the virus, which represents a MOI between 100 and 1.

When CPE is first observed, 1 or 2 days for EEE and WEE, 4 to 7 days for Langat and dengue, the cells are harvested. With viruses such as LCM and CCHF where CPE is not apparent, the day of harvesting was determined by trial and error, usually from 6 to 8 days. The maintenance medium was drained off,

the monolayer was rinsed with Hanks' balanced salt solution (BSSH) and 10 ml of a mixture of equal parts of 0.25% trypsin and 1:5000 versene was added and incubated at 37C for 3 minutes; the trypsin-versene was poured off and the monolayer further incubated at 37C for an additional 20-30 minutes. The detached cells are picked up in 2 or 3 ml of PBS and gently pipetted up and down a few times to insure even dispersion; cells from 6 to 10 flasks are combined in a pool. The pooled cells are sedimented by centrifugation at 1500 rpm for 5 minutes, washed 3 times in PBS and resuspended in an amount of PBS equal to 2 ml per flask used; this suspension constitutes the master suspension. When dealing with BHK-21 and CER cells only trypsin, no versene was used. A count was made of the cells in the master suspension and the volume adjusted with PBS so as to have 3×10^6 cells/ml; between 3 and 10 ml of adjusted suspension were obtained from a 150 cm^2 flask. A similar suspension was prepared from normal, uninfected cell cultures, which was likewise adjusted to 3×10^6 cells/ml.

Infected and uninfected cell-adjusted suspensions were mixed in proportion of 3 parts infected to 1 uninfected. One drop of the mixed cells' suspension was deposited through a 27-gauge needle mounted on a 0.25 ml tuberculin syringe onto each of 10 spots of a 12-spot slide; such drops have an approximate volume of 0.01 ml, therefore contain about 3×10^4 cells. To the last 2 spots similar drops of uninfected cells were added. This procedure insured that at least one fourth of the cells in each spot were not infected, giving an excellent contrast with the infected cells when stained with the fluorescent dye; and also that control cells alone were present on each slide.

After distributing the cells, the slides were air dried in the warm room at 37C for 30 minutes, then fixed in 2 changes of acetone, 5 minutes each. The slides were stored at -60C in slide boxes inside plastic bags.

Preparation of chamber-slides. Tissue culture slides 75 x 25 mm, with 4 chambers, were used, supplied by Lab-Tek Products, Division of Miles Laboratories, Neperville, Illinois; the capacity of the chambers is 1 ml.

All monolayers under fluid medium were grown by seeding each chamber with 2 to 2.5×10^5 cells in a volume of 0.8 - 0.9 ml of growth medium; the slides were held at 37C in a humidified, 5% CO_2 incubator. After 2 to 4 days depending on the cell -- 2 days for CER, 4 or 5 for VERO -- the monolayer was ready for infection. The fluid was aspirated off and the monolayer inoculated with 0.1 ml of the suspension being tested for virus, in increasing dilutions. Following 1 hour incubation at 37C, 0.8 ml of maintenance medium was added and the slides were held at 37C in the CO_2 incubator. At intervals thereafter as desired, slides were processed for IF; the chambers were removed, the slides were immersed in PBS for 10 minutes, then in 2 changes of acetone each 8 minutes. The slides were air dried and stored at -60C until stained.

Immunofluorescence test. Fluorescent conjugates were purchased from commercial dealers; they are antiglobulin goat serum conjugated with fluorescein isothiocynate (FITC). Antiglobulins for man, mouse, hamster, rabbit and guinea pig have been used. The indirect IF technique used followed a standard procedure (Gardner and McQueen, 1975); Evans' blue at dilution 1:10000 was used as a counter stain. A "Vanox" Olympus incident light microscope with a xenon light source, set for blue fluorescence was used; the slides were observed mainly with the x20 power dry objective and the x100 power glycerol immersion one. Glycerol was placed directly on the slide without a coverslip, which

greatly simplified the operation. Readings were made as: positive (+), very weak positive (±), questionable (?), and negative (0).

Residual virus on the spot-slides. For a 12 spot-slide 0.5 ml of diluent -- 2.5% fetal calf serum in PBS -- was measured into a test tube; part of this diluent was taken up in a 0.25 ml syringe provided with a 20-gauge needle, and a drop or two were deposited on the 10 spots containing infected cells. The spots were thoroughly scraped with the sharp edge of the bevel during 10-15 minutes; the drops with the scrapings were collected with the syringe and needle and added to the left over diluent in the tube. This material designated undiluted sample, was titrated for virulence in increasing ten-fold dilutions by ic inoculation to newborn mice or by inoculation of cell monolayers under fluid medium.

Other serological tests. The methods for CF and HI tests have been described in detail (Casals, 1967); they are semi-micromethods which employ a total volume of reagents of 0.15 ml in 6 drops for the CF test and 0.1 ml in 4 drops for the HI test. Reference antigens for both tests were prepared from infected newborn mouse brain tissue by sucrose-acetone extraction.

Determination of neutralizaing antibodies was done by assay on monolayers under fluid medium in Costar 6 x 4 cluster wells; constant virus and serum dilutions were used.

V. Results

Keeping qualities of spot-slides at various temperatures. Slides, lot #359, were prepared with EEE virus in VERO cells; 10 slides each were stored at 4C, 22C (room temperature) and -60C, the latter being the temperature for routine storage. Slides from each group were tested by indirect IF 22 and 78 days after preparation; mouse hyperimmune antisera for EEE, dengue 2, Eyach and Nyamanini viruses were used, the last three as controls for specificity. Assigning to the slides kept at -60C used as reference a value of 4 in terms of brightness and sharpness of image, the result with the slides at the other temperatures is shown in Table 1.

The titer of the EEE serum was the same, 1:64, with all the slides except those kept at 22C for 78 days, when no IF was apparent; there was no trace of nonspecific IF with any of the slides stained with the control sera. With respect to brightness and sharpness, spot-slides kept at 4C for 22 days were as good, or nearly as good, as those held at -60C; at the later time, 78 days, they were not satisfactory. At 22C, deterioration of the slides proceeded more rapidly than at 4C; they could be used with qualifications after 22 days, but were a total loss at 78 days. A characteristic of the slides stored at 4C for 78 days and even more so when kept at 22C for 22 days was that the antigen detected by IF had a tendency to margination; the fluorescence in the cytoplasm had migrated towards the periphery of the cells and accumulated under the cell membrane, resulting in an image of membranous fluorescence or margination of fluorescence.

One example of the excellent keeping quality of spot-slides at -60C was noted with CCHF virus in CER cells; slides prepared in May, 1977, were used 16 months later. As far as one could tell by comparison with freshly prepared slides, the image and brightness given by the old slides were indistinguishable from those given by the new ones.

Residual infective virus in spot-slides. Previous non-quantitative observations had indicated that active virus was present on spot-slides prepared with JE and VEE viruses. A quantitative determination of infective virus was done with slides prepared with WEE, EEE and chikungunya viruses.

The slides had been prepared 8, 6 and 4 months before testing with EEE, WEE and chikungunya, respectively. The fixed material on the slide was recovered as described under methods. Designating the material recovered in 0.5 ml of diluent as 10° or undiluted, increasing 10-fold dilutions were made and tested by ic inoculation into 1 to 3-day-old mice. The result of the titrations, given in Table 2, shows that the procedure employed for the preparation of the slides followed by long term storage at -60C, does not fully inactivate the virus; evidently, inactivation of the virus is advisable.

Inactivation of spot-slides. In view of the high virus contents of the spot-slides attempts were initiated to explore the possibility of preparing avirulent slides; thus far, these attempts were made only by treating infected cell suspensions with betapropiolactone (BPL) before they are deposited on the slide.

EEE virus. A suspension containing 3×10^6 infected VERO cells/ml, as used in the preparation of spot-slides, was divided in 2 equal parts. To one of the aliquots was added 0.05% BPL, final concentration, and held at 37C; the second aliquot was kept at 4C. After 1 hour, spot-slides were prepared with both suspensions and the suspensions were titrated for infective virus on VERO cell monolayers under fluid medium.

Titration of virulence showed that the suspension not treated with BPL had a titer equal to $10^{9.7}$ $TCID_{50}$/ml, while the BPL-treated had a titer of 10^4 $TCID_{50}$/ml. By IF with a mouse hyperimmune serum, the treated slides had fewer cells, approximately half as many as the untreated. The cells were somewhat distorted and some showed margination of fluorescence; the titer of the serum, however, was the same, 1:64, and equally easy to read.

A large reduction of infectivity was achieved, but not enough to render the slides avirulent. In the IF test, the BPL-treated slides were nearly as good as the non-treated ones in terms of sharpness and brightness of image.

Dengue type 2. An experiment similar to the above was done with LLC-MK2 cells infected with dengue type 2 virus; half the volume of a cell suspension was treated with 0.05% BPL for 1 hour at 37C, the other half was not treated and held at 4C. The virulence of the suspensions was titrated by ic inoculation of 1-day-old mice. No virus was recovered from the BPL-treated preparation, even when inoculated undiluted; the untreated suspension had an $ICLD_{50}$ equal to $10^{-5.7}$/0.02 ml. Staining of the slides with a mouse hyperimmune serum gave the same titer by IF, 1:32; the treated, avirulent slides were somewhat less sharp and bright, although just as easy to read as the untreated.

Polyvalent spot-slides. Polyvalent spot-slides were prepared for the alphavirus genus (group A). Separate VERO cell cultures were infected with one of the following viruses: EEE, WEE, VEE and chikungunya. Different cultures were infected with each virus on two consecutive days, in order to insure that on the 3rd day monolayers would be available for each virus

showing CPE of 1 or 2 plus. The infected cells were processed separately for each virus, standardized to contain 4 x 10^6 cells/ml and mixed in equal volumes to make a suspension containing 1 x 10^6 cells/ml infected with each virus; the mixed suspension was used for preparation of spot-slides in the routine method. The capacity of the slides to detect antibodies against group A viruses was tested with hyperimmune mouse sera and ascitic fluids; in one of the tests, immune reagents for EEE, WEE, VEE, Chickungunya, Una, Mayaro, Semliki and Sindbis were used with the results shown in Table 3.

In this and other tests, all immune reagents reacted strongly, even those for viruses not included in the mixture used for preparing the slides.

Detection of early antibodies in experimentally infected animals. Langat virus in hamsters: The results of in-vitro tests, reported for the most part in the first annual report (5 April 1978), are now completed. Hamsters were inoculated once with an amount of virus equivalent to 10^6 newborn mouse $ICLD_{50}$, by the intraperitoneal (ip) route; they were bled daily for the first 10 days. The results of IF, CF and HI tests are given in Table 4. The sera were first positive by the HI test, with a titer of 1:5, on the 4th day, followed by the IF, with a titer of 1:8 on the 5th day; the CF test gave only traces of positive reaction on the 6th day, but was not clearly positive until the 7th day. Neutralizing antibodies will be tested in the near future.

JE virus in mice: 50-day-old mice were inoculated once by the ip route with 10^6 newborn mouse $ICLD_{50}$ of JE virus (dilution 10^{-3}), bled daily in groups of 3, the sera pooled for each day and stored at -20C until tested. Testing for neutralizing antibodies is completed; the sera were assayed in dilutions against 50 $TCID_{50}$, in VERO cell monolayers under fluid medium, with the result shown in Table 5.

A control normal serum had a non-specific inhibitory action with a titer of 1:9; the sera from the mice given JE virus had a slightly higher neutralizing capacity on days 1 to 3, and a well established protective action by day 4. Antibody determination by IF, CF and HI is pending.

Early detection of antigen. The rationale behind the experiments described in this section is to attempt a rapid diagnosis through early detection of virus or antigen in cell cultures inoculated with suspected infected materials. Exploratory experiments were conducted with known infectious virus suspensions, attempting to determine the correlation between amount of virus inoculated and time of appearance of a positive IF reaction.

Junin virus. VERO cell monolayers under fluid medium in Lab-Tek 4-chambered slides were inoculated with increasing ten-fold dilutions of a virus stock suspension, the $ICLD_{50}$ of which in newborn mice was $10^{-6.5}/0.02$ ml. At daily intervals after inoculation, slides were processed for indirect IF testing with a Junin virus mouse antiserum at dilution 1:8. The result of the experiment is shown in Table 6. Twenty-four hours after inoculation of as little as 1.2 x 10^2 $ICLD_{50}$ of virus (dilution 10^{-5}) a weak but definite positive reaction was observed; at 3 days, a positive reaction was discernible even after inoculation of 1 $ICLD_{50}$. In contrast, CPE was not noticeable until the 4th day and then at a minimal degree. The procedure allowed identification of a small amount of virus 3 or 4 days before CPE was visible.

Dengue type 2 virus. An experiment similar to that described in the preceeding paragraph was done with dengue type 2 virus, NGB strain, in LLC-MK2 cells. Lab-Teck slides were inoculated with increasing ten-fold dilutions of a mouse brain tissue stock having an $ICLD_{50}$ of $10^{-7.0}$ in newborn mice. Slides for IF were processed on days 1, 2, 3, 4 and 7; the results are shown in Table 7. A weak positive IF reaction was seen on day 2 with the slides inoculated with dilutions 10^{-2} and 10^{-3}; the reaction was fully developed on day 3, with a 4 plus intensity in dilutions 10^{-2}, 10^{-3} and 10^{-4}, weaker with dilution 10^{-5}. There was no positive IF reaction at dilution 10^{-6} or higher. CPE was not present during the first 4 days, was complete or nearly so on the 7th day, but only in dilutions 10^{-2}, 10^{-3} and 10^{-4}. In this experiment the IF procedure allowed a definite positive diagnosis on day 3 after inoculation, 4 days before CPE was observed.

Rapid diagnosis of arbovirus infection: Banzi virus in mice. Intraperitoneal inoculation of small amounts of Banzi virus to adult mice results in disease with an incubation period of 7-8 days, followed by death 1 or 2 days later. Since in our experience this economical model of infection and disease is highly reproducible, an experiment has been undertaken through which are investigated approaches to early diagnosis.

Twenty 60-day-old mice were ip inoculated 0.3 ml of a 10^{-7} dilution of Banzi virus, strain H 336; this inoculum contained about 150 newborn mouse $ICLD_{50}$ of virus. The inoculated mice remained well until the 7th day after inoculation at which time 2 of 6 mice left were questionably sick; on the 8th day, 2 of the 4 remaining mice were dead, the other 2 very sick. Each day after inoculation 2 mice were exsanguinated by cardiac puncture under deep ether anesthesia. Thick blood smears were prepared individually from each mouse, fixed by heat followed by either acetone or methyl alcohol, and stored at -60C. The rest of the blood was ejected, half of it (about 0.4 - 0.5 ml) into a test tube containing 0.8 ml of distilled water, the remaining into a dry tube, for clotting and serum separation; for collection of hemolyzed blood and serum, the bloods from the 2 daily sacrificed mice were pooled. The hemolyzed bloods and sera were stored at -60C.

The purpose of the experiment is to test the serial daily specimens for: presence and titer of virus in whole blood; antibodies in serum by HI, CF, IF and neutralization tests; to determine whether Banzi antigen can be detected in the blood smears by indirect or direct IF; and to determine presence of virus in the blood by infecting VERO cell monolayers with blood dilutions and processing infected monolayers daily for IF, as described above for Junin and dengue type 2 viruses. The completed parts of the experiment are: titration of virus in the blood and preparation but not staining for IF of chamber slides inoculated with daily collected bloods.

Titration of blood virus: viremia curve. This has been done by ip inoculation of 1 ml (one ml) of blood, in dilutions, to 30-40 day-old mice. Owing to the limited amounts available, the dilutions were begun at 10^{-2}; bloods that showed no virus at this dilution were inoculated again at dilution 10^{-1}, but only in an amount of 0.5 ml. The result of the viremia titration is shown in Table 8.

No virus was demonstrable the first day after inoculation; beginning with day 2 and until the 5th day, viremia with a titer between $10^{2.9}$ and $10^{3.5}$ was observed, during which period the mice appeared perfectly normal.

The virus titer dropped sharply by the 6th day; and on day 7, when 2 of 6 mice were questionably sick, and day 8 when they were nearly moribund, no virus was detected. In our experience, ip inoculation of Banzi virus to adult mice is as sensitive a method as any for detecting the virus.

Immunofluorescence test with human sera. Investigations with human sera have continued to prove the practical applicability of the IF to diagnostic problems and seroepidemiological surveys with arboviruses and arenaviruses.

Lassa fever virus. The survey to detect immune persons among hospital staff personnel in West Africa, particularly Liberia, conducted in association with Dr. John D. Frame, College of Physicians and Surgeons, Columbia University, New York, N.Y., has continued through 1978. Spot-slides bearing Lassa fever virus infected cells were generously supplied by Dr. Karl M. Johnson, CDC, Atlanta, Ga.

Between 15 April and 15 December 1978, 278 sera from residents in Liberia, Niger and Rwanda were screened at dilution 1:4 only: 6 sera were positive, 3 gave a $\pm$ reaction, i.e., positive but borderline; 8 were questionable, probably negative; and 261 were negative. All the positive sera were from Liberia.

CCHF virus. A small survey for IF antibodies against this virus was done in association with Dr. O. Imami, Pristina, Yugoslavia; 70 sera from lifetime residents of Serbia were screened at dilution 1:4. The sera were from currently healthy persons, who had no recollection of a severe hemorrhagic disease; 3 were positive.

Pichinde virus. In the first annual report (5 April 1978) it was stated that 7 of 22 persons associated with a research laboratory in which work with Pichinde and other arenaviruses was being conducted, had IF and CF antibodies against Pichinde virus. Additional serum samples were received shortly after that report was submitted, for a grand total of 58 sera from 45 individuals.

The sera were screened at dilution 1:4 by IF against Pichinde antigen; a number were also tested against Tacaribe and Junin, but positives were found only against Pichinde. All positive sera at dilution 1:4 were titrated to determine their end-points; sera that at dilution 1:4 gave a questionable or weak reaction with readings of $\pm$, 1 and 2 plus, were retested beginning at dilution 1:2, or even undiluted. The sera were also tested by CF in increasing two-fold dilutions beginning at 1:4, extending to 1:32, against several antigens, including Pichinde, Tacaribe, normal tissue and two other viruses not arenaviruses. Positives by CF were found only for Pichinde virus, with one exception to be described later. The combined results of these tests, including those given in part in the previous annual report, are shown in Table 9.

In the IF test, 17 sera were positive with titers between 1:8 and 1:128; 14 of these same sera and none negative by IF, were also positive by CF with titers from 1:4 to 1:32 or higher. Only one serum, #35, reacted with both Pichinde and Tacaribe, with titers of 1:8 and 1:4 respectively.

Serum #35 derived from an investigator, G.G., who has been ill with clinical manifestations during the course of his work; as he was at the time engaged on work with Tacaribe virus, it was assumed that he had been infected with this agent. Additional serum samples from this worker were supplied by Dr. Geoff Gard, Australia, and tested by IF and CF against

Pichinde and Tacaribe antigens; the results of the tests are given in Table 11. These results indicate that G.G. suffered two infections in succession; the first one with Tacaribe (or a closely related virus) between April and November 1976; and the second with Pichinde (or a closely related agent) between December 1976 and October 1977.

Supply of spot-slides for IF to USAMRIID. As part of the contract agreement, spot-slides were prepared, tested for specificity and adequacy and shipped to USAMRIID. The viruses and cells used and the number of slides supplied were:

chikungunya, Ross strain, VERO: 200
Dengue 2, NGB, LLC-MK2; 400
Naples phlebotomus fever, Sabin strain, VERO; 200
Polyvalent group A, VERO; 200
WEE, McMillan strain, VERO; 200
Yellow fever, Asibi strain, VERO; 200

VI. Discussion

The need for early diagnosis of certain arbovirus and arenavirus diseases is predicated on the grounds that effective measures can be taken that are beneficial to the treatment of the patient, and that may prevent or minimize serious consequences to other individuals, provided that the specific etiology is established soon. Person-to-person transmission has been well documented with a number of these viruses, Lassa, Machupo, CCHF, VEE, possibly also with Rift Valley fever, as well as with others not in these sets but in some respects similar, Marburg and Ebola.

Early diagnosis of these diseases can be attempted basically in two ways: virus identification and antibody determination. Since the problem is vast with many ramifications, a large effort would be required to attack it at all points. The present research was planned with the goal to elucidate an aspect of rapid diagnosis, namely, whether a relatively new technique, IF, could be profitably used for early diagnosis of arbovirus and arenavirus diseases.

Investigations thus far carried out have demonstrated that it is feasible to prepare and store adequate numbers of microscope slides -- spot-slides -- bearing on their surface fixed cells infected with a variety of arboviruses and arenaviruses. Using these slides as antigen, antibodies can be rapidly detected and identified by immunofluorescence. It is particularly important that polyvalent slides can be prepared by mixing cell cultures individually infected with different viruses; when polyvalent slides are available for various groups of viruses, it will be possible to test in a step-wise fashion sera from patients with an unknown febrile illness suspected of being caused by an arbovirus or arenavirus; this procedure should accelerate the diagnosis.

An aspect in the preparation of spot-slides is still and to a considerable extent unresolved; in order to make these slides completely safe, means to inactivate them without deteriorating the quality must be developed.

The efficacy of the IF test as a rapid diagnostic method has to be compared with that of other serological tests now in common use, neutralization,

HI and CF, as well as with others that are being developed for use with these viruses, radioimmunoassay and enzyme linked immunoassays. Since field specimens may not be generally available when needed, an experimental approach has been devised, based on developing infection models using small laboratory animals. These models allow the collection of serum samples daily beginning immediately after infection, to be examined by the several tests.

Detection of virus rather than antibody is the optimal procedure for early diagnosis, provided it can be done directly in clinical specimens and that it is not a time-consuming procedure. Some of the infection models described in this report may give an experimental approach to this question.

VII. Conclusions

1. On the basis of observations with EEE virus in VERO cells, spot-slides can be satisfactorily stored at 4C for short periods of time, 3 weeks or somewhat longer; afterwards the slides tend to deteriorate, although they may be usable at 75 days. At a higher temperature, 22C, the slides are usable for at least 7 or 8 days but have appreciably deteriorated at 22 days.

2. Infective virus can be recovered in large amounts from spot-slides prepared by fixation with acetone and stored at -60C for up to 9 months.

3. Preparation of satisfactory avirulent spot-slides by treatment of infected cell suspensions with betapropiolactone may be possible; more research is needed on this subject.

4. Polyvalent slides can be prepared for arbovirus group A that react with all immune sera from the group with which they have been tested.

5. A rapid and specific identification of Junin and dengue 2 virus infection can be done by inoculating with stock virus suspensions -- presumably also with naturally infected materials -- cell monolayers in chamber-slides, followed by daily observation of slides by indirect IF; from 2 to 4 days is gained over detection of CPE.

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Table 1

Immunofluorescence test with EEE virus on spot-slides stored at different temperatures

Length of storage, days	Temperature of storage; result		
	-60C	4C	22C
22	4	4	2
78	4	2	0

Performance rating in a scale from 4 to 0.
The titer of an EEE serum was 1:64 with all the slides rated 4 and 2.
Three control sera for, dengue 2, Eyach and Nyamanini viruses, gave no nonspecific reaction at dilutions 1:4 and higher.

Table 2

Recovery of infective virus from
spot-slides prepared with VERO cells

Virus	$1CLD_{50}/0.02ml$
EEE	$10^{-5.4}$
WEE	$10^{-3.0}$
Chikungunya	$10^{-3.0}$

ic titrations in 1 to 3-day-old mice.

Table 3

Immunofluorescence test with group A polyvalent slides

Antiserum	Titer
EEE	1:8+
WEE	1:8+
VEE	1:8+
Chikungunya	1:8+
Una	1:8+
Mayaro	1:8+
Semliki	1:8+
Sindbis	1:8

Table 4

Antibody development in hamsters given a single intraperitoneal inoculation of Langat virus, 10^6 newborn mouse $ICLD_{50}$

Serum, day	Test: reciprocal of titer IF	CF	HI (8 units)
2	0	0	0
4	0	0	5
5	8	AC	40
6	8	TR	10
7	32	64	80
8	64	64	80
9	64	64	80
10	32	32	40

AC, anticomplementary; TR, traces or partial fixation at dilutions 1:2 to 1:8.

First dilution of serum: IF, undiluted; CF, dilution 1:2; HI, dilution 1:5.

Table 5

Neutralizing antibody development in mice given a single intraperitoneal inoculation of Japanese encephalitis virus, 10^6 newborn mouse $ICLD_{50}$

Serum, day	Titer against 50 $TCID_{50}$
Control	1:9
1	1:35
2	1:42
3	1:55
4	1:93
5	1:97
7	1:100
8	1:290
9	1:336
11	1:244

Assay in VERO cell monolayers, under fluid medium.

Table 6

Relationship between amount of Junin virus inoculated and time of appearance of a positive IF reaction in VERO cell monolayers

Inoculum, 0.1 ml		Days after inoculation											
Dilution of stock	Newborn mouse $ICLD_{50}$	1		2		3		4		5		7	
		IF	CPE	IF	CPE	IF	CPE	IF	CPE	IF	CPE	IF	CPE
10^{-2}	1.2×10^{5}	1	0	1	0	2	±	4	3	4	3		
10^{-3}	1.2×10^{4}	1	0	1	0	2	±	4	1	4	1		
10^{-4}	1.2×10^{3}	1	0	1	0	1	±	4	1	4	1		
10^{-5}	1.2×10^{2}	1	0	1	0	1	0	4	1	4	1	4	2-3
10^{-6}	1.2×10^{1}	±	0	±	0	1	0	4	1	4	1	4	2-3
10^{-7}	1.2	0	0	0	0	1	0	3	0	4	±	4	2-3

IF: in a scale of 4, maximum, to 0,none.

CPE: in a scale of 4, complete destruction of monolayer, to 0, none.

Table 7

Relationship between amount of dengue type 2 virus inoculated and time of appearance of a positive IF reaction in LLC-MK2 cell monolayers

Inoculum, 0.1 ml		Days after inoculation									
Dilution of stock	Newborn mouse $ICLD_{50}$	1		2		3		4		7	
		IF	CPE	IF	CPE	IF	CPE	IF	CPE	IF	CPE
10^{-2}	5×10^{5}	?	0	1	0	4	0	4	0		4
10^{-3}	5×10^{4}	0	0	1	0	4	0	4	0		4
10^{-4}	5×10^{3}	0	0	0	0	4	0	4	0		3
10^{-5}	5×10^{2}	0	0	0	0	1	0	1	0		0
10^{-6}	5×10^{1}	0	0	0	0	0	0	0	0	0	0
10^{-7}	5	0	0	0	0	0	0	0	0	0	0
10^{-8}	0.5	0	0	0	0	0	0	0	0	0	0

Footnote, see Table 6.

Table 8

Banzi virus: viremia determinations in mice IP inoculated with 150 $ICLD_{50}$ of virus, bled on successive days after inoculation

Bled, day after inoculation	Result of inoculation						
	Dilution of blood inoculated						Titer, LD_{50}/ml
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	
1	0*	0					$10^{0.5}$ or less
2		6	5	1	0	0	$10^{3.5}$
3		6	2	1	0	0	$10^{2.9}$
4		6	3	1	0	0	$10^{3.1}$
5		5	3	0	0	0	$10^{2.9}$
6	2	0	0				$10^{0.9}$
7	0	0					$10^{0.5}$ or less
8	0	0					$10^{0.5}$ or less

* Mice dead of 6 inoculated.

Table 9

IF and CF tests results with sera from individuals exposed to Pichinde virus in the laboratory.

Serum, no.	Pichinde antigen: Test and reciprocal of titer		Serum, no.	Pichinde antigen: Test and reciprocal of titer	
	IF	CF		IF	CF
1	0	NS	30	0	0
2	16	NS	31	64	8
3	128	32+	32	128	32
4	128	32+	33	0	0
5	0	NS	34	0	0
6	0	NS	35	16	8
7	0	0	36	0	0
8	128	32+	37	0	0
9	0	NS	38	0	0
10	128	32+	39	8	0
11	0	0	40	0	0
12	0	0	41	0	0
13	0	0	42	8	4
14	0	0	43	0	0
15	16	8	44	0	0
16	0	0	45	0	NS
17	0	0	46	32	8
18	0	0	47	0	0
19	64	32+	48	0	0
20	0	0	49	0	0
21	0	0	50	0	0
22	0	0	51	0	0
23	8	4	52	0	0
24	0	0	53	0	0
25	0	0	54	64	4
26	0	0	55	0	0
27	128	32+	56	16	0
28	0	0	57	0	0
29	0	0	58	0	0

0, negative reaction at dilution 1:2 or 1:4.

NS, non-specific.

Table 10

Results of immunofluorescence and complement-fixation test with serial sera from an individual exposed to Pichinde and Tacaribe viruses in the laboratory

Serum, bleeding date	Test, antigen, reciprocal of titer			
	IF		CF	
	Pichinde	Tacaribe	Pichinde	Tacaribe
06/04/75	0	0	AC	AC
10/27/75	0	0	0	0
04/08/76	0	0	0	0
11/19/76	0	16	0	4
12/07/76	0	16	0	4
10/12/77	16	4	8	2-4
06/20/78	8	4	8	2-4

0, negative reaction at dilution 1:2.

AC, anticomplementary.

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